Pestic. Sci. 1998, **54**, 179–181

Extended Summary Physicochemical and Biophysical Panel of the SCI Pesticides Group and the Royal Microscopical Society Meeting: Microlocalisation of Chemicals within Biological Systems

The following extended summary is based on a paper presented at the above meeting organised by D. Bartlett and N. Read on behalf of the Physicochemical and Biophysical Panel of the SCI Pesticides Group and the Royal Microscopical Society, held on 10 September 1997 at Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, UK. This is entirely the responsibility of the authors and does not necessarily reflect the views of the Editorial Board of Pesticide Science.

Imaging Spitzenkörper, pH and Calcium Dynamics in Growing Fungal Hyphae

Nick D. Read, Sabine Fischer & Richard M. Parton

Fungal Cell Biology Group, Institute of Cell and Molecular Biology, University of Edinburgh, Rutherford Building, Edinburgh EH9 3JH, UK

Plant diseases caused by filamentous fungi result in the loss of crops worth billions of pounds per year. Agrochemical companies are therefore continually trying to discover and develop new and effective antifungal compounds which prevent or inhibit the growth of fungal pathogens in order to improve crop quality and yield.[1] The mechanistic basis of the growth of filamentous fungi is currently the focus of much research. A major challenge in the future will be to discover novel antifungal targets within the multifactorial process of fungal growth.

Tip growth is the main way by which fungi grow, involving the polarized extension of a hypha by means of localized secretion and cell-wall synthesis at the hyphal apex.[2,3] In Edinburgh we are using confocal microscopy with a range of vital fluorescent probes to analyse hyphal tip growth and its regulation in living cells.[4,5] Three aspects being concentrated on are: (1) Spitzenkörper behaviour; (2) intracellular pH; and (3) intracellular Ca^{2+}.

Growing hyphal tips contain a multi-component organelle assemblage called the Spitzenkörper which is assumed to contain the secretory vesicles responsible for tip growth. Spitzenkörper behaviour is associated intimately with the dynamic growth pattern and morphogenesis of the hyphal tip.[5–11] We have developed a fluorescent staining procedure which allows the dynamic behaviour of the Spitzenkörper to be visualised (in growing hyphae) for the first time.[5]

The Spitzenkörper-selective dye we use is FM4-64. This styryl dye stains components of the endocytotic pathway and also, after membrane recycling, exocytotic vesicles. FM4-64 (and related dyes especially FM1-43) has been extensively used to image and analyse membrane trafficking during endocytosis and exocytosis in animal cells,[12] and more recently has been used to study endocytosis in yeast.[13,14] Using this dye we have stained the Spitzenkörper in growing hyphae of a wide range of filamentous fungi including *Aspergillus niger. v.* Teig, *Neurospora crassa* Shear & Dodge, *Puccinia graminis* Pers., *Rhizoctonia solani* Kuhn, *Sclerotium rolfsii* Sacc. and *Trichoderma viride* Pers. (Fischer, S., Rentel, M. C., Hickey, P., Dijksterhuis, J. & Read, N. D., unpubl.). At appropriate concentrations the dye is non-cytotoxic to hyphae and, compared with other dyes, relatively resistant to photobleaching.[5]

We found that the organization and behaviour of Spitzenkörper in stained hyphae of *N. crassa*, *R. solani* and *T. viride* visualized by confocal microscopy closely resembled that in unstained hyphae imaged by computer-enhanced, phase-contrast microscopy.[5] The dynamic shape of the stained Spitzenkörper and its close association with sites of localized cell expansion within the growing hyphal tip are clearly evident (Fig. 1). Hyphal branch formation can be readily monitored

 Pestic. Sci. 0031–613X/98/$17.50. Printed in Great Britain

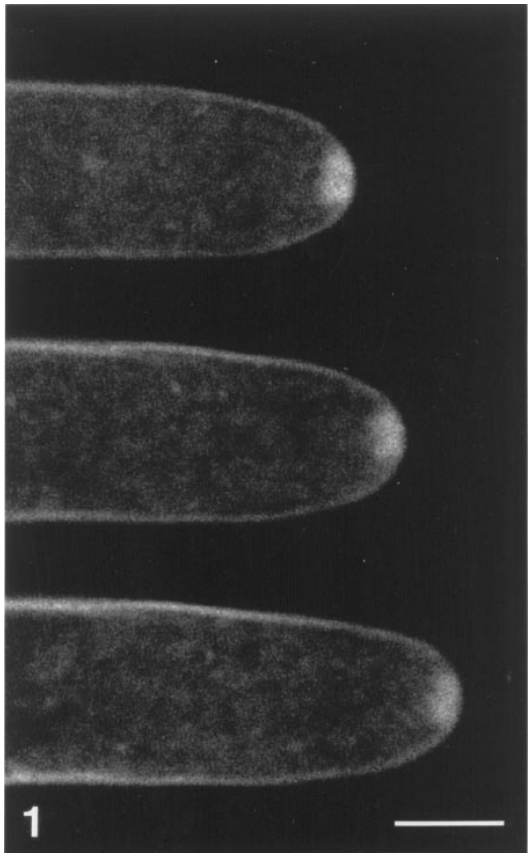

Fig. 1. *Neurospora crassa.* Confocal images of a hyphal tip at three stages of growth showing staining of the Spitzenkörper with FM4-64. Bar, 5 μm.

and a differentiated core region within the Spitzenkörper is often seen. Satellite Spitzenkörper, which arise several micrometres behind the main Spitzenkörper and subsequently fuse with it, are frequently visible (Fig. 2). Staining also allows the effects of environmental stress (e.g. UV light) on Spitzenkörper behaviour and tip growth to be examined.[5] A kinesin-deficient mutant of *N. crassa*, recently reported to lack a Spitzenkörper,[15]

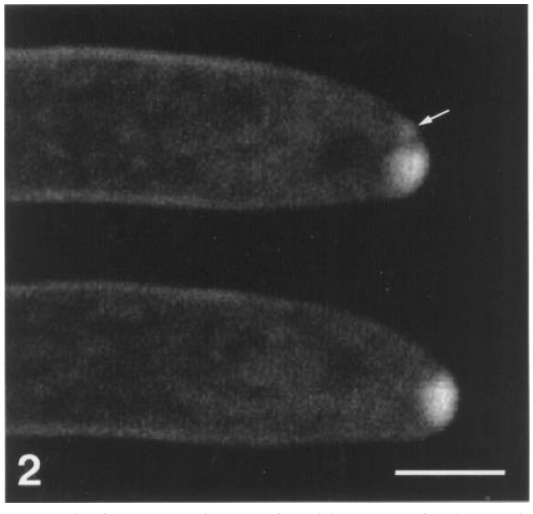

Fig. 2. *Trichoderma viride.* Confocal images of a hyphal tip at two stages of growth showing staining of the main Spitzenkörper and a satellite Spitzenkörper (arrow). Bar, 5 μm.

has been found to contain one which was smaller and more labile than the Spitzenkörper of wild-type hyphae.[5] Overall, it is clear that the combination of confocal microscopy and FM4-64 staining will provide a new and powerful tool for analysing the role of the Spitzenkörper and vesicle trafficking during hyphal tip growth.[5]

Gradients in cytosolic pH and Ca^{2+} have both been implicated as playing important roles in regulating tip growth in the hyphae of higher fungi, and a number of studies involving dye imaging have reported the existence of these gradients.[16–19] Unfortunately, none of this published work has provided convincing evidence supporting the existence of cytosolic pH or Ca^{2+} gradients in higher fungal hyphae because insufficient controls were performed to eliminate the potential problems of imaging artefacts. These artefacts include those resulting from dye partitioning from the cytosol into organelles, altered behaviour of the dye within the cellular microenvironment, and poor dye loading into cells.[4,20–22] Over the last few years we have developed improved methods for the imaging and measurement of cytosolic pH and Ca^{2+} in hyphae using ion-sensitive fluorescent dyes and confocal microscopy.

To analyse cytosolic pH in *N. crassa* we performed confocal ratio imaging of hyphae loaded with the pH-sensitive fluorescent dye carboxy SNARF-1.[4] Using this approach, cytosolic pH could be measured with a precision of $\pm 0{\cdot}3$ to $\pm 0{\cdot}06$ pH unit and a spatial resolution of equal to or better than 2·3 μm^2. Based on in-vitro calibrations, estimated values of the mean cytosolic pH were 7·20–7·25 in hyphae loaded with dye-ester and 7·57 in hyphae loaded with dextran-conjugated dye. Dextran-dyes are believed to provide better estimates of cytosolic pH because of their superior localization and retention within the cytosol, although pressure injection of dextran-conjugated dyes is much more difficult to perform than other dye loading techniques. Pronounced longitudinal cytosolic pH gradients (Δ 0.1 pH unit) were absent from the apical 50 μm of growing *N. crassa* hyphae loaded with either dye-ester or dextran-dye. Further evidence against pronounced cytosolic pH gradients being essential for tip growth was obtained when hyphae continued growing after their cytoplasm had been either acidified or alkalinized with a cell-permeant weak acid or base. These treatments would be expected to collapse pronounced cytosolic pH gradients. Nevertheless, cytosolic pH is normally tightly regulated because changes in external pH resulted in only small transient changes in cytosolic pH. External pH can regulate the initiation of cell polarity because elevating the external pH from 5·7 to 8·0 induced increased branch formation.[4]

Imaging cytosolic free Ca^{2+} has proved considerably more difficult than imaging cytosolic pH in the hyphae of higher fungi. Ester-loading Ca^{2+}-sensitive dyes was mostly unsuccessful and ionophoretic injection of free

dye resulted in extreme sequestration of dye within organelles, especially the pleiomorphic vacuolar network of fungal hyphae.[20,21] To overcome these problems of dye sequestration, we recently used pressure injection to introduce the Ca^{2+}-sensitive, 10 kDa dextran conjugate of Oregon Green-1 into growing hyphae (Fischer, S. & Read, N. D., unpubl.). Initial results clearly demonstrate excellent retention of the dye within the cytosol and the loaded dye can be shown to be highly responsive to changes in cytosolic free Ca^{2+} when hyphae are treated with a high concentration of extracellular Ca^{2+}. Tip-focused gradients in cytosolic free Ca^{2+} have not yet been observed but this may simply be due to the fact that a single wavelength dye has been used. Ion gradients are best observed if ratio imaging with dextran-conjugated ratiometric dyes, or ratiometric dye pairs, is performed. The problem with single wavelength dyes is that it is difficult to distinguish between differences in ion concentration and variations in dye brightness caused by factors such as variable dye concentration, dye photobleaching or dye leakage from the cytosol. In principle, ratio measurements are independent of the amount of dye fluorescence measured and proportional to the free ion concentration, allowing for improved calibration of ion concentration.[20,22]

It is now well established that both Ca^{2+} signalling and pH homeostasis are extremely important for tip growth in fungi and both may offer potentially useful targets for novel antifungal compounds.[23]

REFERENCES

1. Dixon, G. K., Copping, L. G. & Hollomon, D. W. (eds), *Antifungal Agents. Discovery and Mode of Action.* βios Scientific Publishers, Oxford, 1985.
2. Heath, I. B., *Tip growth in Plant and Fungal Cells.* Academic Press, San Diego, 1990.
3. Gow, N. A. R., Tip growth and polarity. In *The Growing Fungus*, ed. N. A. R. Gow & G. M. Gadd. Chapman & Hall, London, 1995, pp. 277–99.
4. Read, N. D., Fischer, S., López-Franco, R., Rentel, M. C. & Bracker, C. E., Fluorescent staining of the Spitzenkörper: evidence for endocytosis and membrane recycling during hyphal tip growth. Submitted.
5. Parton, R. M., Fischer, S., Malhó, R., Papasouliotis, O., Jelitto, T. C., Leonard, T. & Read, N. D., Pronounced cytoplasmic pH gradients are not required for tip growth in plant and fungal cells. *J. Cell Sci.*, **110** (1997) 1187–98.
6. López-Franco, R., Bartnicki-García, S. & Bracker, C. E., Pulsed growth in fungal hyphal tips. *Proc. Natl Acad. Sci. USA*, **91** (1994) 12228–32.
7. López-Franco, R., Howard, R. J. & Bracker, C. E., Satellite Spitzenkörper in growing hyphal tips. *Protoplasma*, **188** (1995) 85–103.
8. Bartnicki-García, S., Bartnicki, D. D., Gierz, G., López-Franco, R. & Bracker, C. E., Evidence that Spitzenkörper behaviour determines the shape of a fungal hypha: a test of the hyphoid model. *Exp. Mycol.*, **19** (1995) 153–9.
9. López-Franco, R. & Bracker, C. E., Diversity and dynamics of the Spitzenkörper in growing hyphal tips of higher fungi. *Protoplasma*, **195** (1996) 90–111.
10. Bracker, C. E., Murphy, D. J. & López-Franco, R., Laser microbeam manipulation of cell morphogenesis in growing fungal hyphae. In *Proceedings of Functional Imaging and Optical Manipulation of Living Cells.* Society of Photo-Optical Instrumentation Engineers, Bellingham, Washington, vol. 2983, 1997, pp. 67–80.
11. Reynaga-Peña, C. G., Gierz, G. & Bartnicki-García, S., Analysis of the role of the Spitzenkörper in fungal morphogenesis by computer simulation of apical branching in *Aspergillus niger*. *Proc. Natl Acad. Sci. USA*, **94** (1997) 9096–101.
12. Betz, W. J., Mao, F. & Smith, C. B., Imaging exocytosis and endocytosis. *Curr. Opinion Neurobiol.*, **6** (1996) 365–71.
13. Vida, T. A. & Emr, S. D., A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.*, **128** (1995) 779–92.
14. Reider, S. E., Banta, L. M., Köhrer, K., McCaffery, J. M. & Emr, S. D., Multilamellar endosome-like compartment accumulates in the yeast vps28 vacuolar protein sorting mutant. *Mol Biol. Cell*, **7** (1996) 985–99.
15. Seiler, S., Nargang, F. E., Steinberg, G. & Schliwa, M., Kinesin is essential for cell morphogenesis and polarized secretion in *Neurospora crassa*. *EMBO J.*, **16** (1997) 3025–34.
16. Turian, G., Cytochemical gradients and mitochondrial exclusion in the apices of vegetative hyphae. *Experientia*, **35** (1979) 1164–6.
17. Roncal, T., Ugalde, U. O. & Irastorza, A., Calcium-induced conidiation in *Penicillium cyclopium*: calcium triggers cytosolic alkalinization at the hyphal tip. *J. Bacteriol.*, **175** (1993) 879–86.
18. Levina, N. N., Lew, R. R., Hyde, G. J. & Heath, I. B., The roles of Ca^{2+} and plasma membrane ion channels in hyphal tip growth of *Neurospora crassa*. *J. Cell Sci.*, **108** (1995) 3405–17.
19. Robson, G. D., Prebble, E., Rickers, A., Hosking, S., Denning, D. W., Trinci, A. P. J. & Robertson, W., Polarized growth of fungal hyphae is defined by an alkaline pH gradient. *Fungal Genet. Biol.* **20** (1996) 289–98.
20. Read, N. D., Allan, W. T. G., Knight, H., Knight, M. R., Malhó, R., Russell, A., Shacklock, P. S. & Trewavas, A. J., Imaging and measurement of cytosolic free calcium in plant and fungal cells. *J. Microsc.*, **166** (1992) 57–86.
21. Knight, H., Trewavas, A. J. & Read, N. D., Confocal microscopy of living fungal hyphae microinjected with Ca^{2+}-sensitive fluorescent dyes. *Mycol. Res.*, **97** (1993) 1505–15.
22. Parton, R. M. & Read, N. D., Calcium and pH imaging in living cells. In *Light Microscopy in Biology—A Practical Approach*, 2nd edn, ed. A. J. Lacey. IRL Press, Oxford, 1998 (in press).
23. Read, N. D., Collis, A. J., Fischer, S. & Jelitto, T. C., Calcium signalling and pH homeostasis as novel targets for antifungal compounds. In *Antifungal Agents. Discovery and Mode of Action*, ed. G. K. Dixon, L. G. Copping & D. W. Holloman. βios Scientific Publishers, Oxford, 1995, pp. 279–83.